

(51) International Patent Classification <sup>4</sup> : <b>C12N 15/13</b>		A1	(11) International Publication Number: <b>WO 89/ 01974</b>
			(43) International Publication Date: 9 March 1989 (09.03.89)
(21) International Application Number: PCT/GB88/00730 (22) International Filing Date: 5 September 1988 (05.09.88) (31) Priority Application Number: 8720833 (32) Priority Date: 4 September 1987 (04.09.87) (33) Priority Country: GB (71) Applicant (for all designated States except US): CELL-TECH LIMITED [GB/GB]; 216 Bath Road, Slough, Berkshire SL1 4EN (GB). (72) Inventors; and (75) Inventors/Applicants (for US only) : BODMER, Mark, William [GB/GB]; 131 Reading Road, Henley-on-Thames, Oxfordshire R619 1DJ (GB). ADAIR, John, Robert [GB/GB]; 23 George Road, Stokenchurch, High Wycombe HP14 3RN (GB). WHITTLE, Nigel, Richard [GB/GB]; 5 Leigh Road, Cobham, Surrey KT11 2LF (GB).		(74) Agent: MERCER, Christopher, Paul; Carpmaels & Ransford, 43 Bloomsbury Road, London WC1A 2RA (GB). (81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, RO, SE (European patent), SU, US. Published <i>With international search report.          Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: RECOMBINANT ANTIBODY			
(57) Abstract			
The present invention provides an altered antibody molecule (AAM) having a hinge region which has a different number of cysteine residues from that found in the hinge region normally associated with the CH1 domain of the antibody molecule and a process for producing the same using recombinant DNA technology.			

***FOR THE PURPOSES OF INFORMATION ONLY***

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	ML	Mali
AU	Australia	GA	Gabon	MR	Mauritania
BB	Barbados	GB	United Kingdom	MW	Malawi
BE	Belgium	HU	Hungary	NL	Netherlands
BG	Bulgaria	IT	Italy	NO	Norway
BJ	Benin	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	LI	Liechtenstein	SN	Senegal
CH	Switzerland	LK	Sri Lanka	SU	Soviet Union
CM	Cameroon	LU	Luxembourg	TD	Chad
DE	Germany, Federal Republic of	MC	Monaco	TG	Togo
DK	Denmark	MG	Madagascar	US	United States of America
FI	Finland				

- 1 -

## Recombinant antibody

The present invention relates to an altered antibody molecule (AAM) having an altered number of cysteine residues in its hinge region and to a process for its production using recombinant DNA technology.

In the present application:

the term "MAb" is used to denote a monoclonal antibody;

the term "recombinant antibody molecule" (RAM) is used to describe an antibody produced by any process involving the use of recombinant DNA technology, including any analogues of natural immunoglobulins or their fragments; and

the term "humanised antibody molecule" (HAM) is used to describe a molecule having an antigen binding site derived from an immunoglobulin from a non-human species, the remaining immunoglobulin-derived parts of the molecule being derived from a human immunoglobulin. The antigen binding site may comprise either complete variable domains fused onto constant domains or only the complementarity determining regions (CDRs) grafted onto appropriate framework regions in the variable domains.

In the description, reference is made to a number of publications by number. The publications are listed in numerical order at the end of the description.

Natural immunoglobulins have been known for many years, as have the various fragments thereof, such as the Fab, (Fab')<sub>2</sub> and Fc fragments, which can be derived by enzymatic cleavage. Natural immunoglobulins comprise a generally Y-shaped

- 2 -

molecule having an antigen-binding site at the end of each arm. The remainder of the structure, and particularly the stem of the Y, mediates the effector functions associated with immunoglobulins.

At the junction of the arms of the Y-shaped molecule, there is an area known as the hinge region. In this region there are, depending on the class of the antibody, between 2 and 11 inter-heavy chain disulphide bonds. These disulphide bonds are responsible for holding together the two parts of the complete antibody molecule. In a Fab fragment, the hinge region has been enzymatically separated from the antigen binding region. Thus, the Fab fragment comprises a light chain/truncated heavy chain dimer. However, in the  $(\text{Fab}')_2$  fragment, the Fc portion is cleaved off the antigen binding regions on the C-terminal side of the hinge region. Thus, the  $(\text{Fab}')_2$  fragment comprises two light chain/truncated heavy chain dimers held together in a tetrameric structure by the hinge region.

The hinge region allows the arms of the Y-shaped antibody molecule to move relative to one another. It is conjectured that the degree of movement is determined to a large extent by the number of disulphide bonds in the hinge region. It is also believed that the hinge region plays a key role in the transmission of conformational changes from the antigen binding regions to the Fc portion of the molecule. Such conformational changes may be necessary in order to activate the effector functions of the immunoglobulin molecule.

In natural antibodies, in particular the IgGs, the hinge represents a distinct region of the antibody, at both the protein and gene level. High resolution data on the in vivo conformation of the

- 3 -

hinge region are not available from the structural studies performed to date. However, model building suggests that the hinge regions may form a relatively simple extended or helix-like structure linked by disulphide bridges.

Natural immunoglobulins and their fragments have been used in diagnosis and, to a more limited extent, in therapy. However, such uses, especially in therapy, have been hindered by the polyclonal nature of natural immunoglobulins. A significant step towards the realisation of the potential of immunoglobulins as therapeutic agents was the discovery of monoclonal antibodies (1) of defined antigen specificity. Most MAbs are produced by fusions of rodent spleen cells with rodent myeloma cells. They are therefore essentially rodent MAbs. There are very few reports of the production of human MAbs.

There have been made proposals for "humanizing" rodent MAbs. These techniques generally involve the use of recombinant DNA technology to manipulate DNA sequences encoding the polypeptide chains of the antibody molecule.

Some early methods for carrying out such a procedure are described in EP-A-0 171 496 (Res. Dev. Corp. Japan), EP-A-0 173 494 (Stanford University), EP-A-0 194 276 (Celltech Limited) and WO-A-8 702 671 (Int. Gen. Eng. Inc.).

In an alternative approach, described in EP-A-87302620.7 (Winter), the complementarity determining regions (CDRs) of a mouse MAb have been grafted onto the framework regions of the variable domains of a human immunoglobulin by site directed mutagenesis using long oligonucleotides.

- 4 -

It has been widely suggested that immunoglobulins, and in particular MAb's, could potentially be very useful in the diagnosis and treatment of cancer (2,3). There has therefore been much activity in trying to produce immunoglobulins or MAb's directed against tumour-specific antigens. So far, over one hundred MAb's directed against a variety of human carcinomas have been used in various aspects of tumour diagnosis or treatment (4).

In our copending, concurrently filed application No. (PA 149) there is described a humanised antibody molecule (HAM) having an antigen binding site wherein at least the complementarity determining regions (CDRs) of the variable domain are derived from the mouse monoclonal antibody B72.3 (B72.3 MAb) and the remaining immunoglobulin-derived parts of the HAM are derived from a human immunoglobulin, and a process for its production.

The B72.3 MAb is a mouse MAb of the type IgG1-Kappa raised against a membrane-enriched extract of a human liver metastasis of a breast carcinoma (5). The B72.3 MAb has been extensively studied in a number of laboratories. It has been shown to recognise a tumour-associated glycoprotein TAG-72, a mucin-like molecule with a molecular weight of approximately  $10^6$  (6). Immunohistochemical studies have demonstrated that the B72.3 MAb recognises approximately 90% of colorectal carcinomas, 85% of breast carcinomas and 95% of ovarian carcinomas. However, it shows no significant cross-reactivity with a wide spectrum of normal human tissues (7 to 10).

It has been suggested, for instance in EP-A-0 194 276, that effector or reporter molecules

- 5 -

may be attached to an antibody or a fragment thereof in order to increase its effectiveness as a therapeutic or diagnostic agent. The attachment may be by means of a covalent bridging structure. Alternatively, where the effector or reporter molecule is a protein, it may be coexpressed as the C-terminal part of a fusion protein, the N-terminal part of which comprises at least the variable domain of one of the chains of the antibody molecule or fragment.

In all the work carried out so far, the hinge region, if present, in the antibody molecule or fragment has been that normally associated with the CH1 domain of the antibody molecule. There has been no suggestion that the hinge region should in any way be altered except in so far as it may be necessary to alter its C- or N-terminal sequence to facilitate manipulations by recombinant DNA technology.

According to a first aspect of the present invention, there is provided an altered antibody molecule (AAM) having a hinge region which has a different number of cysteine residues from that found in the hinge region normally associated with the CH1 domain of the antibody molecule.

The AAM of the present invention may comprise: a complete antibody molecule, having full length heavy and light chains; an (Fab')<sub>2</sub> fragment; or any other fragment including a hinge region. The antigen binding portions of the AAM may, if desired, have different specificities, the antibody in this case being bispecific.

The AAM of the present invention may have attached to it an effector or reporter molecule. For instance, the AAM may have a macrocycle, for chelating a heavy metal atom, or a toxin, such as

- 6 -

ricin, attached to it by a covalent bridging structure. Alternatively, the procedures of recombinant DNA technology may be used to produce a AAM in which the Fc fragment or CH<sub>3</sub> domain of a complete antibody molecule has been replaced by an enzyme or toxin molecule.

Preferably, the AAM of the present invention is also a HAM, for instance of the type described in the above-referenced copending application. The variable domains of the HAM may comprise either the entire variable domains of a rodent MAb or may comprise the framework regions of a human variable domain having grafted thereon the CDRs of the rodent MAb. The remainder of the HAM may be derived from any suitable human immunoglobulin. However, it need not comprise only protein sequences from the human immunoglobulin. For instance, a gene may be constructed in which a DNA sequence encoding part of a human immunoglobulin chain is fused to a DNA sequence encoding the amino acid sequence of a polypeptide effector or reporter molecule.

The altered hinge region of the AAM of the present invention may comprise a complete hinge region derived from an antibody of different class or subclass from that of the CH<sub>1</sub> domain. Thus, for instance, a CH<sub>1</sub> domain of class  $\gamma$  1 may be attached to a hinge region of class  $\gamma$  4. Alternatively, the new hinge region may comprise part of a natural hinge or a repeating unit in which each unit in the repeat is derived from a natural hinge region. In a further alternative, the natural hinge region may be altered by converting one or more cysteine residues into neutral residues, such as alanine, or by converting suitably placed residues into cysteine residues. It can thus be seen that the number of cysteine residues in the hinge region may be increased or decreased.



- 7 -

In one preferred aspect of the invention, the number of cysteine residues in the hinge region is reduced to one. This will have the advantage that it will facilitate assembly of the antibody molecules, particularly bispecific antibody molecules and antibody molecules wherein the Fc portion has been replaced by an effector or reporter molecule, since it will only be necessary to form a single disulphide bond. This will provide a specific target for attaching the hinge region either to another hinge region or to an effector or reporter molecule, either directly or indirectly, by chemical means.

In an alternative preferred aspect, the number of cysteine residues in the hinge is increased. The advantage of this is that it will facilitate the use of the cysteine thiol groups for attaching effector or reporter molecules to the AAM. For instance, <sup>99m</sup> technecium, a radiolabel, may be attached to hinge cysteines either directly or by use of a macrocycle ligand as described in our copending British applications Nos. 8800843 and 8812257. Increasing the number of cysteines can also be used to stabilize the interactions between adjacent hinges.

It will be appreciated by the skilled person that the present invention enables him to make an informed trade-off between improved specificity of attachment and assembly on the one hand and improved stability and decreased specificity on the other hand.

Preferably, the AAM of the present invention is produced by recombinant DNA technology. Therefore, according to a second aspect of the present invention, there is provided a process for producing an AAM according to the first aspect of the invention, which process comprises:

(a) producing in an expression vector an operon which includes a DNA sequence encoding an antibody heavy chain having a hinge region which has a different number of cysteine residues from that found in the hinge region normally associated with the CH1 domain of the antibody molecule.

The operon may be produced by splicing a DNA sequence encoding the CH1 region from an antibody of one class to a DNA sequence encoding the hinge region from an antibody of a different class.

Alternatively, the operon may be produced by cloning the CH1 domain and hinge region from an antibody of one class and altering the number of cysteine residue encoding DNA triplets by site directed mutagenesis. Preferably, where the number of cysteine residues is to be decreased, the cysteine-encoding sequences are mutated to alanine-encoding sequences. Where the number of cysteine-encoding sequences is to be increased, any suitably positioned non-cysteine residue may be altered.

Preferably, the process of the second aspect of the invention includes the steps of:

(b) transfecting a cell line with the vector;  
and

(c) culturing the transfected cell line to produce the AAM.

In the process of the second aspect of the present invention, as the vector encodes only the heavy chain antibody polypeptide it will be necessary to arrange for the cell line to produce a complementary light chain. In order to achieve this, one of three alternative strategies may be employed.

In the first alternative, the cell line may be transfected with a second vector, the second vector

encoding a complementary light chain-derived polypeptide. Preferably, the vectors are identical except in so far as the coding sequences and selectable markers are concerned so as to ensure as far as possible that each polypeptide chain is equally expressed.

In the second alternative, the vector may include sequences coding for both light chain- and heavy chain-derived polypeptides.

In the third alternative, it is possible to produce the AAM by using a host cell which naturally secretes a complementary light chain.

The present invention also includes cloning and expression vectors and transfected cell lines used in the process of the invention, therapeutic and diagnostic compositions containing the AAM of the invention and uses of such compositions in therapy and diagnosis.

The general methods by which the vectors may be constructed, transfection methods and culture methods are well known per se and form no part of the invention. Such methods are shown, for instance, in references 11 and 12.

The present invention is now described, by way of example only, with reference to the accompanying drawing which shows the natural amino acid sequences of the four human IgG hinge regions.

In our above-referenced copending, concurrently filed application No. (PA 149) we describe in detail the production of humanized antibody molecules based on the B72.3 MAb and in particular, the construction of whole antibody molecules and F(ab') fragments is shown. The application describes the construction of various expression vectors and their use in producing

- 10 -

humanised B72.3 antibodies (B72.3 HAMs). This work forms the basis for the work now described.

Natural human IgGs can have one of four possible hinge regions. The amino acid sequences of these hinge regions are shown in the accompanying drawing. From the known amino acid sequence it will readily be possible for the skilled person to design or mutate oligonucleotides to encode these sequences, or variants thereof with cysteine additions or deletions.

#### EXAMPLE 1

In Example 3 of the PA 149 application, there is described an (Fab')<sub>2</sub> molecule having the B72.3 variable domains and a human IgG4 CH1 domain and hinge region. In order to encourage selective reduction at the hinge of this B72.3 HAM and to reduce the complexity of subsequent chemical additions at the hinge, the number of cysteines in the B72.3 HAM hinge region (which is normally two) was reduced to one by replacing the second hinge cysteine residue with an alanine residue.

A part of the amino acid sequence of the hinge region and its corresponding DNA encoding sequence is shown below.

CCC	CCA	TGC	CCA	TCA	TGC	CCA
Pro	Pro	Cys	Pro	Ser	Cys	Pro

- 11 -

Modified B72.3 Chimaeric IgG4(Fab')<sub>2</sub>Single Cysteine IgG4 HingeConstruction and Assembly of Modified F(ab') Gene

To construct a modified hinge onto the end of the CH1 domain in pJA108 (described in our copending application PA149), four oligonucleotides were made which together are able to code for the last five amino acids of the CH1 domain, the hinge sequence, two inframe stop codons and an EcoRI site.

The oligonucleotides required to form the new Cys-Ala modified hinge sequence have the following sequences:

1

5'TCGACAAGAGACTTGAGTCCAAATATGGG

3'GTTCTCTCAACTCAGGTTTATACCCGGGGG

2

3

5'CCCCCGTGCCCATCAGCCCCATGATG

3'CACGGGTAGTCGGGGTACTACTTAA

4

Oligonucleotides 1 and 3 were used to form the sense strand and oligonucleotides 2 and 4 were used to form the anti-sense strand.

The modified CH1/hinge region junction encoded by the modified gene has the amino acid sequence:

Lys Val Asp Lys Arg Val Glu Ser Lys Tyr Gly Pro Pro  
Cys Pro Ser Ala Pro Stop Stop

- 12 -

The CH1/hinge region junction is between the Val and Glu residues.

The oligonucleotides were assembled and cloned into M13mp11 between the SallI and EcoRI sites in the polylinker, sequenced, reisolated and ligated to the gene containing the EcoRI - SallI 700bp fragment from pJA108 to construct the modified (hinge cys to ala) chimaeric B72.3 F(ab') heavy chain gene.

#### Assembly of Gene in Expression Vector

The modified chimaeric B72.3 F(ab') heavy chain gene fragment, assembled as described above, was subsequently cloned into the EcoRI vector fragment of pJA96 to give pJA115.

#### Test of Genes in COS Cells

The genes were tested in COS cells as described in the PA149 application. On non-reducing SDS-PAGE the material appeared to be produced as F(ab') material only. Reducing SDS-PAGE the material appeared to be produced as F(ab') material only. Reducing SDS-PAGE showed the presence of light chain and truncated heavy chain equivalent to that expected from the modified F(ab') gene.

#### Development of Stable Cell Lines in CHO Cells

The expression plasmid pJA115, comprising the hinge modified B72.3 chimaeric F(ab') heavy chain gene fragment expressed from the HCMV promoter, was introduced by electroporation into the CHO cell line cL18 described in the PA149 application. The procedure was similar to that described for

- 13 -

introduction of the full length chimaeric heavy chain except that the SalI digestion was omitted and the DNA was introduced as closed circular DNA. Cell lines resistant to mycophenolic acid and expressing functional modified F(ab') antibody was identified by screening culture supernatants in an antigen binding ELISA format assay as described earlier. Cell lines expressing between 0.1-10 µg/ml modified F(ab') were isolated. One cell line, 18ΔF9, was used for further studies.

CHO cell lines expressing the modified F(ab') were also isolated using gene amplification. A plasmid vector comprising the chimaeric light chain and modified F(ab') heavy chain gene fragment, each expressed separately from the HCMV promoter, and the glutamine synthetase (GS) minigene described in our International patent application No. PCT/GB88/00602, namely pEE6.cH.cL.GS, was introduced into CHO-K1 cells by the calcium phosphate precipitation procedure.

Transfectants were selected in GMEM medium as described in our International patent application No. PCT/GB88/00602 comprising 20 µM methionine sulfoximine (MSX). Cell lines expressing functional modified F(ab') antibody were identified by screening culture supernatants in an antigen binding ELISA format assay as described in our copending application. Cell lines expressing between 0.05-1µg/ml modified F(ab') were obtained and subjected to screening for gene amplification by plating out in MSX concentrations ranging from 30-5000µM. Amplified cell lines growing in medium containing MSX at 200µM were found to express modified F(ab') to a level of 10-20µg/ml. One amplified cell line, 31.2 was used for further studies.

- 14 -

Purification of modified chimaeric F(ab')<sub>2</sub> Antibody

Hinge modified chimaeric F(ab') was purified from CHO cell supernatants using standard procedures. CHO cell culture supernatant containing modified chimaeric F(ab') was adjusted to 60% ammonium sulphate, the precipitate was resuspended in suitable buffer and passed over a DEAE-Sepharose column. Fractions containing F(ab') material were pooled and, after dialysis, passed over S-Sepharose. Fractions containing F(ab') material were pooled and then dialysed extensively into PBS and concentrated by ultrafiltration. By this process, the yield of F(ab')<sub>2</sub> is increased to approximately 40% of the finally purified material.

Hinge modified F(ab') was purified from CHO cell supernatant using immunopurification. An immunopurification reagent was prepared by linking NH3/41, an antibody with specificity for human Kappa chain sequence, to cyanogen bromide activated Sepharose by standard methodology. This material was packed into a column and equilibrated with PBS. CHO cell culture supernatant containing modified chimaeric F(ab') was applied to the column and the column was washed with PBS. Elution of modified chimaeric F(ab') was then achieved using 4.5M guanidine hydrochloride. Fractions containing modified chimaeric F(ab') were then dialyzed extensively into PBS and concentrated by ultrafiltration. In repeat purifications, approximately 10% of the material can be found as F(ab')<sub>2</sub> which forms without further treatment.



- 15 -

Crosslinking of Modified Chimaeric F(ab')<sub>2</sub> Antibody

F(ab')<sub>2</sub> material from mouse B72.3, prepared by enzymatic digestion of whole antibody, and modified chimaeric F(ab') material prepared as described in the preceeding paragraph were chemically crosslinked by linking half-cysteine residues via thioether bonds with the use of the homo-bifunctional cross-linking reagent 1,6 bismaleimidohexane (Sigma). B72.3 F(ab')<sub>2</sub> or modified chimaeric F(ab') at 1-5 mg/ml in 0.15M phosphate buffer pH8.0, containing 2mM EDTA, was reduced by the addition of 2-mercaptoethylamine to a final concentration of 5mM and incubated at 37°C for 30 minutes. After reduction, samples were desalted on a Sephadex G25 column equilibrated with 0.1M citric acid/0.2M Na<sub>2</sub>PO<sub>4</sub> pH6.0/2mM EDTA. The crosslinker was dissolved in dimethylformamide in a final concentration of 72mM and added to the freshly reduced F(ab')SH at a level of 1.9mM (approx. 22 fold excess over titratable thiols) and incubated for 90 minutes with constant mixing. N-ethyl maleimide was added to a final concentration of 9mM and incubated further for 30 minutes before desalting into 0.1M citric acid/0.2M Na<sub>2</sub>PO<sub>4</sub> pH6.0/2mM EDTA. The maleimidated F(ab'), (Fab')(MAL), was immediately added to a fresh batch of F(ab')SH at a molar ratio of 1.1:1.0 and incubated at room temperature with constant mixing for 20 hours.

The composition of the crosslinking reaction was determined by HPLC gel filtration after overnight incubation. 10μl of the reaction mixture was added to 10μl of 200mM 2-mercaptoethylamine and incubated for 15 minutes. A further addition of 20μl of 800mM iodoacetamide was made and incubated for 15 minutes.

- 16 -

The reduced and alkylated sample was then analysed by HPLC GF 250 and the percentage composition of the chromatogram was determined by integration. Material eluting in the position of  $F(ab')_2$  was assumed to be chemically crosslinked  $F(ab')_2$ . The elution times closely matched the expected  $F(ab')_2$  retention times of various standards. The percentage crosslinking was higher for the chimaeric  $F(ab')$  material (one cysteine in the hinge) than for the mouse  $F(ab')$  material (three cysteines in the hinge) and contained less aggregated material. Reduction in the complexity of the hinge in this way may therefore lead to more efficient linking and superior product.

#### EXAMPLE 2

Chimaeric B72.3 IgG4  $F(ab')_2$  (IgG1 Hinge)

#### Construction of $F(ab')$ Heavy Chain Gene

To construct a hinge modified gene, the chimaeric  $F(ab)$  region containing the B72.3VH/IgG4 gene was isolated as a 0.7 kbp fragment from JA 108 (described in our copending patent application PA 149) by treating the DNA with Sall, removing the 5' phosphate from the Sall site with calf intestinal phosphatase (CIP), and recutting the DNA with EcoRI.

The IgG1 hinge was assembled by preparing appropriate oligonucleotides to encode the IgG1 hinge. 500 pm of top and bottom strand oligonucleotide were kinase labelled and annealed by heating to 70°C and cooling to room temperature in the kinase buffer. The hinge fragments were ligated to the 0.7 kbp fragment from JA108 prepared as above, and the CIP'ed 5' ends were kinased.

- 17 -

Assembly of Gene in Expression Vector

The chimaeric B72.3 F(ab') heavy chain gene fragment, assembled as described above, was subsequently cloned into the EcoRI/CIP treated vector of JA96 to give TR001. Expression of TR001 in suitable cells with an expression vector capable of producing a useful light chain, for example chimaeric or humanised B72.3, will produce material which will assemble to give F(ab') and which will on suitable post translational modification in vivo or in vitro give F(ab')<sub>2</sub>.

EXAMPLE 3

Chimaeric B72.3 IgG4 F(ab')<sub>2</sub> (IgG2 Hinge)

Construction of F(ab') Heavy Chain Gene

To construct a hinge modified gene, the chimaeric F(ab) region containing the B72.3VH/IgG4 was isolated as described in Example 2. The IgG2 hinge was assembled by kinase labelling 500 pm of top and bottom strand oligonucleotide and annealing the oligonucleotides by heating to 70°C and cooling to room temperature in the kinase buffer. The hinge fragments were ligated to the 0.7 kbp fragment from JA108 prepared as above, and the CIP'ed 5' ends were kinased.

Assembly of Gene in Expression Vector

The chimaeric B72.3 F(ab') heavy chain gene fragment, assembled as described above was subsequently cloned into the EcoRI/CIP treated vector

- 18 -

fragment of JA96 to give TR003. Expression of TR003 in suitable cells with an expression vector capable of producing a useful light chain, for example chimaeric or humanised B72.3, will produce material which will assemble to give F(ab') and which will on suitable post translational modification in vivo or in vitro give F(ab')<sub>2</sub>.

#### EXAMPLE 4

Modified B72.3 Chimaeric IgG4 F(ab')<sub>2</sub> (IgG3 Hinge)

#### Construction of Modified F(ab') Heavy Chain Gene

The IgG3 hinge is normally found as a reduplicated 4 exon structure which codes for a repeating sequence of the form

ELKTPLGDTTHTCPRC[PEPKSCDTPPPCPRC]<sub>n</sub>P

where n normally is 3. Hinges of this form can be derived by correct ligation of the following oligonucleotides to the CH1 domain.

1. 5'TCGACAAGAGAGTTGAGCTCAAACCCAC
2. 3'GTTCTCTCAATCGAGTTTGGGGTGAACC
3. 5'TTGGTGACACACAACCTCACACATGCCACGGTG
4. 3'ACTGTGTTGAGTGTGTACGGGTGCCACGGGT
5. 5'CCCAGAGCCCAAATCTTGTGACACACCTCCCCCGTGCCACGGTG
6. 3'CTCGGGTTTAGAACACTGTGTGGAGGGGGCACGGGTGCCACGGGGT
7. 5'CCCATGATG
8. 3'ACTACTTAA

- 19 -

By suitable manipulation of the molar ratios of the oligonucleotides,  $n$  can be varied from 0 upwards to produce hinges of variable but predictable length and sequence.

To construct the IgG3 hinge, oligonucleotides were assembled by kinase labelling and annealing as in the previous Examples and ligating the relevant oligonucleotides to produce the correctly designed product. Because of the possibility of producing concatamers of the central oligonucleotide pair, the ligation products were cloned into pSP64 at the EcoRI and SalI sites. Miniprep DNA was prepared and cut with EcoRI, 3' labelled by filling the EcoRI end with  $^{32}\text{P}$  dATP using the Klenow polymerase, recutting the DNA with SalI and examining the length of the cloned inserts of polyacrylamide gels. A clone containing an insert of the required size to code for the IgG3 hinge was identified, the clone was grown up and the hinge insert isolated on a preparative scale. The hinge fragment was ligated to the 0.7 kbp fragment from JA108 prepared as above and the CIP'ed 5' ends were kinased to enable subsequent recloning.

#### Assembly of Gene in Expression Vector

The chimaeric B72.3 F(ab') heavy chain gene fragment, assembled as described above, was subsequently cloned into the EcoRI/CIP treated vector fragment of JA96 to give TR007. Expression of TR007 in suitable cells with an expression vector capable of producing a useful light chain, for example chimaeric or humanised B72.3, will produce material which will assemble to give F(ab') and which will on suitable post translational modification in vivo or in vitro give F(ab')<sub>2</sub>.

- 20 -

EXAMPLE 5

Modified B72.3 Chimaeric IgG4 F(ab')<sub>2</sub> (IgG3 2 cys Hinge)

Construction of F(ab') Heavy Chain Gene

The IgG3 (2 cys) hinge length variant was assembled by kinase labelling and annealing as above and ligating the relevant oligonucleotides to produce the correct length product. The ligated products were cloned into pSP64 at the EcoRI and SalI sites. Miniprep DNA was prepared and cut with EcoRI, 3' labelled by filling the EcoRI end with <sup>32</sup>P dATP using the Klenow polymerase, recutting the DNA with SalI and examining the length of the cloned inserts on polyacrylamide gels. A clone containing an insert of the required size to code for the IgG3(2 cys) hinge was identified, the clone was grown up and the hinge insert isolated on a preparative scale. The hinge fragments were ligated to the 0.7 kbp fragment from JA108 and the CIP'ed 5' ends were kinased to enable subsequent recloning.

Assembly of Gene in Expression Vector

The chimaeric B72.3 F(ab') heavy chain gene fragment, assembled as described above, was subsequently cloned into the EcoRI/CIP treated vector fragment of JA96 to give TR004. Expression of TR004 in suitable cells with an expression vector capable of producing a useful light chain, for example chimaeric or humanised B72.3, will produce material which will assemble to give F(ab') and which will on suitable post translational modification in vivo or in vitro give F(ab')<sub>2</sub>.

- 21 -

EXAMPLE 6

Modified B72.3 Chimaeric IgG4 F(ab')<sub>2</sub> (IgG3 5 cys Hinge)

Construction of F(ab') Heavy Chain Gene

The IgG3 (5 cys) hinge length variant was assembled by kinase labelling annealing and ligating the relevant oligonucleotides as above to produce the correct length product. Because of the possibility of producing concatamers of the central oligonucleotide pair, the ligation products were cloned into pSP64 at the EcoRI and Sall sites. Miniprep DNA was prepared and cut with EcoRI 3' labelled by filing the EcoRI end with <sup>32</sup>P dATP using the Klenow polymerase, recutting the DNA with Sall and examining the length of the cloned inserts on polyacrylamide gels. A clone containing an insert of the required size to code for the IgG3 (5 cys) hinge length variant was identified, the clone was grown up and the hinge insert isolated on a preparative scale. The ligated products were cloned into pSP64 at the EcoRI and Sall sites. The hinge fragments were ligated to the 0.7 kbp fragment from JA108 prepared as above and the CIP'ed ends were kinased to enable subsequent recloning.

Assembly of Gene in Expression Vector

The chimaeric B72.3 F(ab') heavy chain gene fragment, assembled as described above, was subsequently cloned into the EcoRI/CIP treated vector fragment of JA96 to give TR005 in suitable cells with

- 22 -

an expression vector capable of producing a useful light chain, for example chimaeric or humanised B72.3, will produce material which will assemble to give F(ab') and which will on suitable post translational modification in vivo or in vitro give F(ab')<sub>2</sub>.

#### EXAMPLE 7

Modified B72.3 Chimaeric IgG4 F(ab')<sub>2</sub> (IgG3 8 cys Hinge)

#### Construction of F(ab') Heavy Chain Gene

The IgG3(8 cys) hinge length variant was assembled by kinase labelling annealing and ligating the relevant oligonucleotides as above to produce the correct length product. Because of the possibility of producing concatamers of the central oligonucleotide pair, the ligation products were cloned into pSP64 at the EcoRI and SalI sites. Miniprep DNA was prepared and cut with EcoRI 3' labelled by filling the EcoRI end with <sup>32</sup>P dATP using the Klenow polymerase, recutting the DNA with SalI and examining the length of the cloned inserts on polyacrylamide gels. A clone containing an insert of the required size to code for the IgG3(8 cys) hinge length variant was identified, the clone was grown up and the hinge insert isolated on a preparative scale. The ligated products were cloned into pSP64 at the EcoRI and SalI sites. The hinge fragments were ligated to the 0.7 kbp fragment from JA108 prepared as above and the CIP'ed ends were kinased to enable subsequent recloning.



- 23 -

Assembly of Gene in Expression Vector

The chimaeric B72.3 F(ab') heavy chain gene fragment, assembled as described above, was subsequently cloned into the EcoRI/CIP treated vector fragment of JA96 to give TR006 in suitable cells with an expression vector capable of producing a useful light chain, for example chimaeric or humanised B72.3, will produce material which will assemble to give F(ab') and which will on suitable post translational modification in vivo or in vitro give F(ab')<sub>2</sub>.

It will be appreciated that the present invention has been described above by way of illustration only, and that variations or modifications of detail can be made without departing from the scope of the invention.

- 24 -

References

1. Kohler & Milstein, Nature, 265, 495-497, 1975.
2. Ehrlich, P., Collected Studies on Immunity, 2, John Wiley & Sons, New York, 1906.
3. Levy & Miller, Ann.Rev.Med., 34, 107-116, 1983.
4. Schlom & Weeks, Important Advances in Oncology, 170-192, Wippincott, Philadelphia, 1985.
5. Colcher et al., PNAS, 78, 3199-3203, 1981.
6. Johnson et al., Cancer Res., 46, 850-897, 1986.
7. Stramignoni et al., Int.J.Cancer, 31, 543,552, 1983.
8. Nuti et al., Int.J.Cancer, 29, 539-545, 1982.
9. Thor et al., J.Nat.Cancer Inst., 76, 995-1006, 1986.
10. Thor et al., Cancer Res., 46, 3118-3124, 1986.
11. Maniatis et al., Molecular Cloning, Cold Spring Harbor, New York, 1982.
12. Primrose and Old, Principles of Gene Manipulation, Blackwell, Oxford, 1980.

- 25 -

CLAIMS

1. An altered antibody molecule (AAM) having a hinge region which has a different number of cysteine residues from that found in the hinge region normally associated with the CH1 domain of the antibody molecule.
2. The AAM of claim 1, when produced by recombinant DNA technology.
3. The AAM of claim 1 or claim 2, which is a complete antibody molecule or an F(ab')<sub>2</sub> fragment.
4. The AAM of claim 3, which is bispecific.
5. The AAM of any one of claims 1 to 4, having an effector or reporter molecule attached thereto.
6. The AAM of any one of claims 1 to 5, wherein the hinge region comprises a complete hinge region derived from an antibody of different class or subclass from that of the CH1 domain.
7. The AAM of any one of claims 1 to 5, wherein the natural hinge region has been altered by increasing or decreasing the number of cysteine residues.

- 26 -

8. A process for producing a AAM according to claim 2 or any claim dependent thereon, the process comprising:

(a) producing in an expression vector an operon which includes a DNA sequence encoding an antibody heavy chain having a hinge region which has a different number of cysteine residues from that found in the hinge region normally associated with the CH1 domain of the antibody molecule.

9. The process of claim 8, wherein the DNA sequence encoding the hinge region has been altered by site directed mutagenesis.

10. The process of claim 8, wherein the region of the DNA sequence encoding the end of the CH1 domain and the hinge region have been constructed from oligonucleotides.

11. The process of any one of claims 8 to 10, further including the steps of:

(b) transfecting a cell line with the vector;  
and

(c) culturing the transfected cell line to produce the AAM.

12. The process of claim 11, wherein the cell line is also transformed with a second vector containing an operon having a DNA sequence encoding a complementary light chain-derived polypeptide.

13. The process of claim 11, wherein the vector also contains a DNA sequence encoding a complementary light chain-derived polypeptide.

1/1

HINGE AMINO ACID SEQUENCES.

IgG1	EAPKSCDKTH*	TCPPCP	2 cysteine hinge
IgG2	ERKCCVEC PP	CP	4 cysteine hinge
IgG3	ELKTPLGDTT	HTCPRC PERK	SCDTPPPCPR
	CPEPKSCDTP	PPCPRCPEPK	11 cysteine hinge
IgG4	ESKYGPPCPS	CP	2 cysteine hinge

\* This cysteine is bridged to the light chain.

# INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 88/00730

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC <sup>4</sup> :            C 12 N 15/13														
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched <sup>7</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 30%; border-bottom: 1px solid black;">Classification System</td> <td style="border-bottom: 1px solid black;">Classification Symbols</td> </tr> <tr> <td style="padding: 5px;">IPC<sup>4</sup></td> <td style="padding: 5px;">C 12 N; C 12 P</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup></div>			Classification System	Classification Symbols	IPC <sup>4</sup>	C 12 N; C 12 P								
Classification System	Classification Symbols													
IPC <sup>4</sup>	C 12 N; C 12 P													
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b> <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; text-align: left;">Category <sup>*</sup></th> <th style="width: 70%; text-align: left;">Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup></th> <th style="width: 20%; text-align: left;">Relevant to Claim No. <sup>13</sup></th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">A</td> <td>WO, A, 86/01533 (CELLTECH) 13 March 1986 (cited in the application)</td> <td></td> </tr> <tr> <td style="text-align: center; vertical-align: top;">A</td> <td>EP, A, 0146413 (G.D. SEARLE &amp; CO.) 26 June 1985</td> <td></td> </tr> <tr> <td colspan="3" style="height: 200px; vertical-align: middle; text-align: center;">-----</td> </tr> </tbody> </table>			Category <sup>*</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	A	WO, A, 86/01533 (CELLTECH) 13 March 1986 (cited in the application)		A	EP, A, 0146413 (G.D. SEARLE & CO.) 26 June 1985		-----		
Category <sup>*</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>												
A	WO, A, 86/01533 (CELLTECH) 13 March 1986 (cited in the application)													
A	EP, A, 0146413 (G.D. SEARLE & CO.) 26 June 1985													
-----														
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>*</sup> Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>														
<b>IV. CERTIFICATION</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border-bottom: 1px solid black;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="padding: 5px;">16th December 1988</td> <td style="padding: 5px; text-align: center;">11 JAN 1989</td> </tr> <tr> <td style="border-bottom: 1px solid black;">International Searching Authority</td> <td style="border-bottom: 1px solid black;">Signature of Authorized Officer</td> </tr> <tr> <td style="padding: 5px;">EUROPEAN PATENT OFFICE</td> <td style="padding: 5px;">M. VAN MOL </td> </tr> </table>			Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	16th December 1988	11 JAN 1989	International Searching Authority	Signature of Authorized Officer	EUROPEAN PATENT OFFICE	M. VAN MOL				
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report													
16th December 1988	11 JAN 1989													
International Searching Authority	Signature of Authorized Officer													
EUROPEAN PATENT OFFICE	M. VAN MOL													

# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

GB 8800730

SA 24126

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 30/12/88. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8601533	13-03-86	EP-A- 0194276	17-09-86
		GB-A- 2177096	14-01-87
		JP-T- 62500352	19-02-87
EP-A- 0146413	26-06-85	JP-A- 60252500	13-12-85
		US-A- 4751077	14-06-88

Publ. Inform. No. 12/82

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82